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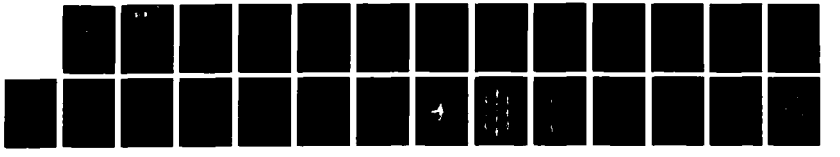

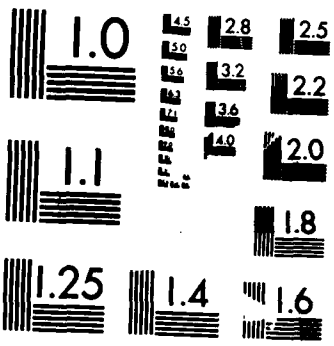

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This review article discusses some of the latest developments and applications in the field of multidimensional fluorescence techniques. Numerous important advances have been made in the past few years, mostly due to improvements in automated instrumentation. In addition, the increased use of multiple parameters has provided recognized advantages. The multiparametric term has been used most often in systems only where the fluorescence intensity is observed as a function of two variables (e.g. excitation and emission). We review the utility of other parameters, including circular dichroism, fluorescence lifetime and chromatographic retention time, in enhancing the fluorescence measurement. In addition, we discuss powerful algorithms which have been developed to extract meaningful information from various types of multidimensional fluorescence data.

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Recent Advances in Multidimensional Fluorescence Spectrometry

by

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INTRODUCTION

Fluorescence spectroscopy has proven to be a valuable analytical technique because of its selectivity and ease of application. The increasing popularity of the technique is reflected in the increasing number of research oriented as well as application oriented publications. Numerous important advances have been made in the past few years, mostly due to improvements in automated instrumentation. In addition, the increased use of multiple parameters has provided recognized advantages.

Although fluorescence is inherently a multiparametric technique, this property has not always been fully exploited. The multiparametric term has been used most often in systems only where the fluorescence intensity is observed as a function of two variables (e.g. excitation and emission). The introduction of additional parameters has obvious advantages with increased selectivity being cited as the most important benefit.

Powerful algorithms have also been developed to extract meaningful information from various types of multidimensional fluorescence data. Several researchers working in the field of chemometrics are devoting significant efforts to developing new mathematical approaches to the analysis of multidimensional fluorescence data. All of the above cited studies clearly demonstrate the utility of multidimensional fluorescence techniques.

This review article discusses some of the latest developments and applications in the field of multidimensional fluorescence techniques. A complete and exhausting review of this important analytical technique is beyond the scope of this manuscript. However, the interested reader may consult several excellent and thorough monographs and review articles (1-5).

Instrumentation

As is usual in modern analytical chemistry, the development of instrumentation is a key factor in determining new directions and exploring new avenues. All areas of chemical analysis have benefited from rapid advances in electronics and computer technology. The availability of powerful, yet affordable, computers have enabled the total automation of analytical instrumentation. The amount and rapidity of information collected during an analysis has also been expanded considerable. This is particularly true for spectroscopic measurements. In the fairly recent past, spectroscopy has often involved relatively tedious and cumbersome measurements resulting in limited information.

Although fluorescence is an inherently selective measurement, most compounds in solutions at room temperature display spectra which are usually very broad and extensive overlap may occur for even simple mixtures. Thus, selectivity in fluorescence measurement can be greatly enhanced by simultaneously measuring several fluorescence properties of the analyte in the same experiment.

Novel advances in data reduction strategies have also encouraged the simultaneous exploitation of multiple parameters. Despite the high degree of computerization, the acquisition of multidimensional fluorescence data can be a time consuming process. There are basically three types of instrumentation available for acquiring fluorescence data as a function of multiple parameters (e.g. excitation and emission). The simplest example is a computer-controlled version of a conventional scanning type fluorometer.[6] A second uses a novel concept of polychromatic illumination coupled with a rapid two dimensional data acquisition detector similar to those found in TV cameras.[7] The third approach utilizes polychromatic imaging of the emission unto a linear diode

array detector system.[8] The development of the latter two types of instrumentation has eliminated specific disadvantages largely associated with conventional scanning types of fluorometers. The most important of these is the time required for acquiring a complete emission-excitation matrix (EEM). The long exposure times of conventional fluorometers would often prevent the examination of photosensitive compounds. But as is often the case with advances, some trade-offs have to be made, such as less sensitivity and higher detection limits for the rapid scanning fluorometers.

The final design of a multidimensional instrument will depend on the desired data acquisition rate and format. For example, if EEMs are to be acquired and the acquisition speed is not a crucial factor, then several excellent commercial instruments are available which use conventional scanning methods. If faster data acquisition is an important goal, the researcher has the option to switch to the vidicon based instrumentation developed in 1975 (7) or to commercially available diode array systems, such as the Tracor Northern TN6100 series diode array detectors. Recent advances in spectroscopy have also made it relatively easy to obtain multiwavelength synchronous spectra (9). In addition, the dimensionality of the data can be increased by the introduction of variables other than the emission or excitation. These new variables can be, but not limited to, fluorescence decay time (10) or chromatographic retention time (11). Similarly, photoselection may also be used to obtain increased dimensionality.[12]

Since the development of instrumentation produced a major impact on the advancement of multidimensional techniques, a short discussion of the different techniques is beneficial to classification and discussion of various multidimensional approaches. Although classification of our topics can be considered to be somewhat arbitrary, this mainly reflects the status of much of the literature on current analytical instrumentation.

A. Total Luminescence Techniques

The simplest fluorescence multidimensional technique involves EEM acquisition, i.e., fluorescence intensity as a function of multiple excitation and emission wavelengths, has been termed total luminescence spectroscopy (TLS). Several well established methods can be chosen for obtaining total luminescence spectra. The most common choice involves the use of more tedious, but usually more sensitive conventional techniques. This task can be simplified through computer control when one of the wavelengths, i.e., excitation or emission, can be fixed and the other scanned. After the required number of repetitions, the desired fluorescence matrix can be obtained and stored in computer memory. Although, this technique was established by Haugen et al.[13] more than a dozen years ago, it is still a frequently used method and several commercially available instruments provide such capabilities.

Alternatively, one can use the somewhat specialized, faster, but usually less sensitive rapid scanning techniques. One of these was first reported in the literature in 1975.[7] In this design, a polychromatic sample illumination was used in conjunction with an analyzing polychromotor and an intensified vidicon camera detector. This instrument design is often referred to as a video fluorometer due to the use of video technology for detection. This system is able to provide the acquisition of a 256 X 256 fluorescence matrix in a time frame as short as 17 msec. Most fluorescence applications do not require this extensive resolution, and thus the size of the matrix is often reduced to a more manageable 64 X 64 format to decrease the data storage requirements.

B. Multidimensional Fluorescence Lifetime Measurements

The temporal dimension of fluorescence can also be incorporated into a multidimensional analysis technique. In recent years, the use of laser excitation has enhanced the use of multidimensional lifetime measurements.

Time resolved emission spectroscopy (TRES) has been used for many years (14,15,16). Although these measurements have potentially approached a multidimensional technique, it was not until the mid-1970's that time and wavelength resolved spectra began to appear in the literature (16). While the acquisition of TRES spectrum (see reference 17 for a review of TRES and its applications) can be a relatively rapid process, acquisition of a time-wavelength resolved emission spectrum could require several hours. An example of this type of spectrum appears in Figure 1.

Time correlated single photon counting (TCSPC) has been the preferred method in obtaining time and wavelength resolved spectra (16,18,19). The spectrum is collected by measuring the fluorescence decay curve at several emission wavelengths. These curves are then deconvoluted from the instrument response function to give the impulse fluorescence response. This step may be accomplished by a variety of data analysis methods which are outside the scope of this review (20). These response functions may then be scaled using steady state emission spectra and plotted against emission wavelength. Alternately, a data collection scheme may be employed which assures that each decay profile was collected using the same number of excitation pulses. This method requires no *a priori* knowledge of the steady state fluorescence spectrum. Several such schemes have been reported (17,19,21). All involve a determination of the measurement time by monitoring the excitation source intensity.

Multidimensional time resolved methods have not been widely used due to the long measurement times required in collecting the data matrix. The use of synchronously pumped dye lasers in TCSPC measurements has been reported to reduce the measurement time by as much as a factor of 50 (19). Recently, a novel technique for acquiring time resolved fluorescence data was described by Hieftje, *et al.* (22,23). This instrument uses a mode locked laser and an

optical delay line which are differentially detected. This detection scheme produces a point on the fluorescence decay curve for each value of optical delay. By scanning an emission monochromator at each delay, a temporally resolved fluorescence spectrum can be generated. Several spectra can be assembled to produce the full time-wavelength resolved spectrum. These types of novel advances will make time-wavelength resolved fluorescence measurements more routine in the study of photophysical phenomena.

An integral part of fluorescence lifetime experimentation is the analysis of decay data. Many types of mathematical schemes have been devised and have been reviewed elsewhere (20). In general, these analysis methods result in a set of functional parameters which best describe the experimental data. A problem arises when these methods are applied to multiple experiments. In such cases, the analysis method cannot take advantage of the information contained in other experiments in the series. A result is produced which is best fit in relation to only one data set.

Global reduction methods have also been recently devised which can analyze multiple data sets simultaneously (24). This global approach can be applied independent of the mathematical form of the fitting procedure. In a global analysis, parameters common to a series of experiments, such as lifetimes, are shared by all the data sets. A goodness of fit statistic is not generated for each experiment, but for the entire series. This has the effect of searching the union of the fitting error surfaces for the individual data sets. The minimum is very sharp at the global minimum, whereas the minima of the individual error surfaces may be ill-defined (24).

Data for global analysis can be obtained in several ways (25). Fluorescence decay spectra can be recorded at different excitation or emission wavelengths, giving data sets of constant lifetime but different intensity

ratios. The method can be applied similarly to anisotropy data. Chemical systems can be observed as a function of other physical parameters, such as pH, temperature, or viscosity. Excited state reactions can be studied as a function of different initial concentrations. Schemes to link functional relationships across different decay curves can be directly incorporated into the data analysis, resulting in better estimates of the parameters of interest.

C. Multidimensional Detection for Chromatography

Increased selectivity in chromatographic detection has been sought since the introduction of chromatographic techniques. Selective detection is especially important when co-eluting analytes are to be identified. Increased dimensionality is one of the possible approaches in these cases. Since fluorescence has long been recognized as a selective chromatographic detector for high performance liquid chromatography (HPLC), the advantages of introducing multidimensional detection schemes are numerous. Thus, this approach increases the resolving power of the modern HPLC system. The traditional scanning type multidimensional detection methods are not feasible in multidimensional HPLC applications due to the dynamic volume of HPLC systems. The objective in designing the liquid chromatographic detector is to detect and capture as much information as is possible in real time. Therefore, rapid scanning multichannel detectors find many applications as a multichannel detector system in HPLC. This increasing use of multichannel detectors, e.g. diode array detectors or video fluorometric detectors is warranted by the increased demand for detecting and identifying complex samples. The utility of this approach has been demonstrated by several authors. The videofluorometer can be cited as one of the most powerful systems for such applications. This approach has been described by Hershberger et al. (26) and in 1979 by Shelly et al. (27). The advantages of this detection method is demonstrated from the EEM detected

chromatogram (Figure 2) of a mixture of PNAs. This approach proved to be very powerful for multicomponent HPLC analysis and its role may increase as more and more complex systems are analyzed.

D. Multidimensional Fluorescence Detected Circular Dichroism

In this method, the measurement of polarization increments the dimensionality by using circularly polarized light for molecular excitation. Since chiral molecules absorb left and right circularly polarized light differently, the detected fluorescence intensity is also different due to the direct proportionality of fluorescence to absorbance. Selectivity is gained from the fact that not every chiral chromophor fluoresces. Additional selectivity is gained in multidimensional fluorescence detected circular dichromision (MFDCD) due to variations in the excitation wavelength and emission wavelengths. Generally, this allows the determination of chiral structures at a particular location in a complex lumophore.

Only a few papers (8,28) have been published related to this relatively new field. Nevertheless, techniques used in MFDCD are very similar to other multidimensional techniques. The utility and the validity of this approach have been demonstrated. This technique is not only being used in structural determinations but also as a solution to physiological, pharmacological and pharmaceutical analytical problems. A typical MFDCD spectrum of cortison appears in Fig. 3B. Figure 3A contrasts the conventional EEM of the same sample.

E. Other Multidimentional Techniques

Several additional variations of multidimensional fluorescence measurements can be conceived by a combination of existing methods. Obviously, the combination of a linear photodiode array detector along with detection of an additional parameter inevitably will result in a multidimentional techniques.

Similarly, any combination of at least two methods results in a multidimensional approach. The analytical possibilities are numerous. Nevertheless, several theoretically possible multidimensional combinations have not yet been exploited.

Data Reduction

The data generated by multidimensional instrumentation require several data reduction steps for meaningful interpretation. Due to the vast amount of data, direct observation of the instrumentation output, either in analog or digital format, is of little value to the researcher. The output in most cases is digital and matrix formatted. Luminescence data in matrix form will generally follow the usual constraints of linear algebra. This phenomena can be explained through the well known properties of the excitation -emission matrix, i.e. for a pure component, the observed emission is independent of the wavelength of exicitation and the excitation spectrum is independent of the monitored emission wavelength (29). It is apparent that this property is also true for emission data acquired as a function of decay time or as a function of chromatographic retention time. Weber was first to recognize the mathematical implications of these properties (30). This early study and a later work (29) have shown that the ideal matrix of luminescence data as a function of multiple excitation and multiple emission wavelengths for a pure component has the linear algebraic form defined above. This is not true for a multicomponent system, since the emission profile changes several times with variations in the excitation wavelength. In fact, the number of changes noted in the emission or excitation profile is an indication of the minimum number of luminescing species contributing to the matrix (29). A number of data reduction algorithms have been developed for the qualitative (29,31) and quantitative (32,33) analysis of such data matrices. The development of these data reduction strategies have permitted the fruitful

application of multidimensional luminescence matrices in many areas of chemistry, including environmental studies (34), clinical studies (35), and physical chemistry (36).

Although different data reduction strategies have been developed for different multidimensional fluorescence methods, depending on the data type, there are noticeable similarities between these algorithms. Accordingly, only a few recent methods will be briefly mentioned here. Most of these methods assume ideal matrices where synergistic effects such as energy transfer are negligible. This is a reasonable assumption under conditions where the absorbance at all wavelengths is low. The basic mathematical format of these ideal matrices will be explained using EEMS as examples, but similar approaches can be applied for other types of multidimensional fluorescence data.

For a pure component, the luminescence matrix, $\underset{\sim}{M}$, is a function of multiple excitation and multiple emission wavelength, and has a linear algebraic form,

$$\underset{\sim}{M} = \alpha \, \underline{x} \, \underline{y} \qquad (1)$$

where α is a concentration dependent parameter, the vectors $\underline{x}$ and $\underline{y}$ represent the excitation and emission spectra of the luminescing component. For an "n" component luminescence mixture, the matrix is a summation of the individual component matrices, i.e.,

$$\underset{\sim}{M} = \sum_{i=1}^{n} \alpha_i \, \underline{x(i)} \, \underline{y(i)} \qquad (2)$$

where i is used to enumerate the components. Fluorescence excitation-emission matrices (EEM) can easily be illustrated by use of three dimensional plots, such as contour or isometric plots. Figure 4 provides a contour plot of a typical one component fluorescence excitation emission matrix. Similarly, Figure 5 provides an EEM contour plot of a three component mixture of polynuclear aromatic hydrocarbons. It should be noted again that the number of changes in

the emission or excitation profile is an indication of the minimum number of fluorescence species contributing to the matrix.

Knorr and Harris (37) have shown that it is possible to decompose the fluorescence emission-time matrix (FETM) into its individual components using the spectra and kinetic behavior of the component fluorophores. This matrix technique is similar in many respects to the methods of Love and Shaver (38). Their one dimensional analogy of the matrix technique performs a reiterative deconvolution of the decay curves at each of the emission wavelengths. In the work of Love and Shaver, luminescence intensity is acquired as a function of emission wavelength and fluorescence decay time. A two dimensional data matrix is formed, and decomposed into two factors: the spectral behavior and the time behavior of the components based on independent first order decay of the spectral components.

Another technique has been described by Goeringer and Pardue (39) which uses different regression methods to extract the rate constants, lifetimes and initial intensity values. A time independent ratio of the intensity of any wavelength can be defined relative to the peak intensity. A ratio deconvolution algorithm has also been used to resolve different components of a mixture of PNAs using multidimensional phosphorimetry (40). This study successfully proved that time resolved phosphorescence-emmision-time matrix (PETM) and ratio deconvolution is a feasible approach to separation of a complex mixture of components having significant spectral overlap.

The utility of increased dimensionality is amply demonstrated when fluorophores have significant overlap in the excitation and emission spectra. Polarization may be used to resolve overlapping bands from different molecules and may even be used to distinguish different electronic transitions from the excited state (41). Although this data processing is not multidimensional, it

is clear that the utility of this approach may be improved by also varying the excitation wavelength. Thus, multidimensional polarization data in the form of depolarization versus excitation and emission wavelength should be extremely useful. A typical example of polarization data which is readily acquired in a multidimensional format is data generated from fluorescence detected circular dichroism (FDCD). As discussed earlier, the FDCD matrix is similar to the EEM acquired by the video fluorometer (42). This analog implies that the mixture resolution algorithms developed for the EEM should also be applicable. However, the ellipticity matrix has legitimate negative values, while the EEM is, theoretically, a non-negative matrix. Thus, the use of different constraints for the resolution algorithm is required. A unique algorithm has recently been described for resolution of the FDCD matrix (42). The effect of spectral overlap on this resolution is also predicted.

As mentioned earlier, several additional parameters can be introduced to increase the selectivity of multidimensional luminescence methods, e.g. chromatographic retention time. In general, the data reduction strategies for these data sets are similar to those previously described, although the data density can be increased enormously. For example, in the video fluorometric detection for HPLC, large quantities of data are generated. Since the three dimensional data sets are generated as a function of chromatographic retention time, the data are truly four dimensional. Appellof and Davidson (43) have shown that certain advantages accrue from analysis of these data in a four-dimensional format. A final example in this category is the coupling of fluorescence lifetime with fluorescence detection of HPLC (44). In this example, data are presented in a three-dimensional display of fluorescence intensity versus chromatographic retention time and luminescence decay time. The utility of this approach has been demonstrated using structurally similar PNAs.

It is obvious that the modern multidimensional approach to fluorescence measurement depends heavily on achievements in data reduction strategies. This need contributes to the ever increasing demand for versatile analytical chemists with expertise in both instrumentation and mathematics.

Conclusion

This short summary of multidimensional methods outlines the advantages of multidimensional luminescence measurements. These advantages arise from the use of a combination of different parameters, e.g. excitation emission and a third parameter (e.g. retention time, lifetime etc.) This greater selectivity has proved particularly useful in mixture analysis. It should be noted, that increasing the dimensionality of fluorescence measurements will generally achieve improved selectivity. However, the desired approach may easily become cumbersome and expensive. A good rule of thumb is always to use the minimum dimensionality necessary to provide the desired analytical information. Powerful, novel computer algoritms have been developed for reduction and interpretation of multidimensional data sets. Use of these algoritms can reduce the number of required experiments and therefore reduce the ultimate cost of the applied method.

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FIGURE CAPTIONS

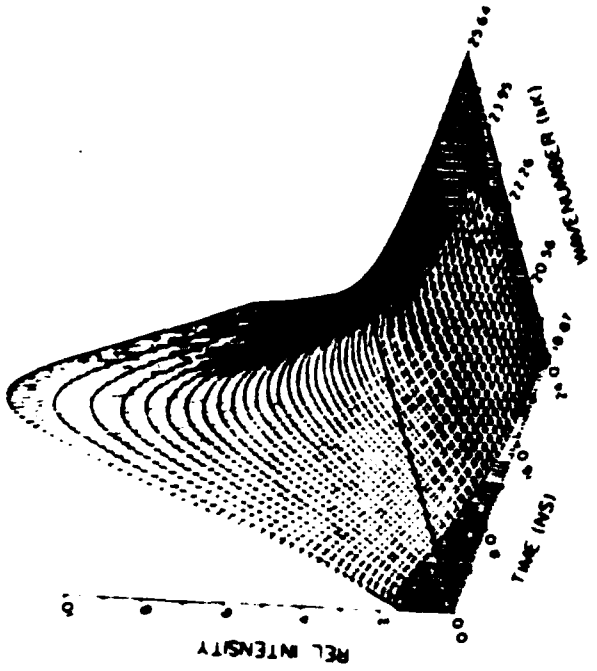
Fig. 1. Time resolved spectra of 2,6p-TNS adsorbed to L-α-egg lecithin vesicles. Reprinted from reference 16.

Fig. 2. The EEM detected HPLC chromatogram of a mixture of PNAs. Reprinted from reference 34.

Fig. 3. a) Typical EEM of cortison in 0.01 M sulfuric acid. b) MFDCD spectra of the same sample.

Fig. 4. Contour plot representation of perylene fluorescence excitation-emission matrix (one component mixture).

Fig. 5. Contour plot representation of the EEM of a three component mixture in cyclohexane (perylene, fluoranthane, and dibenzanthracene).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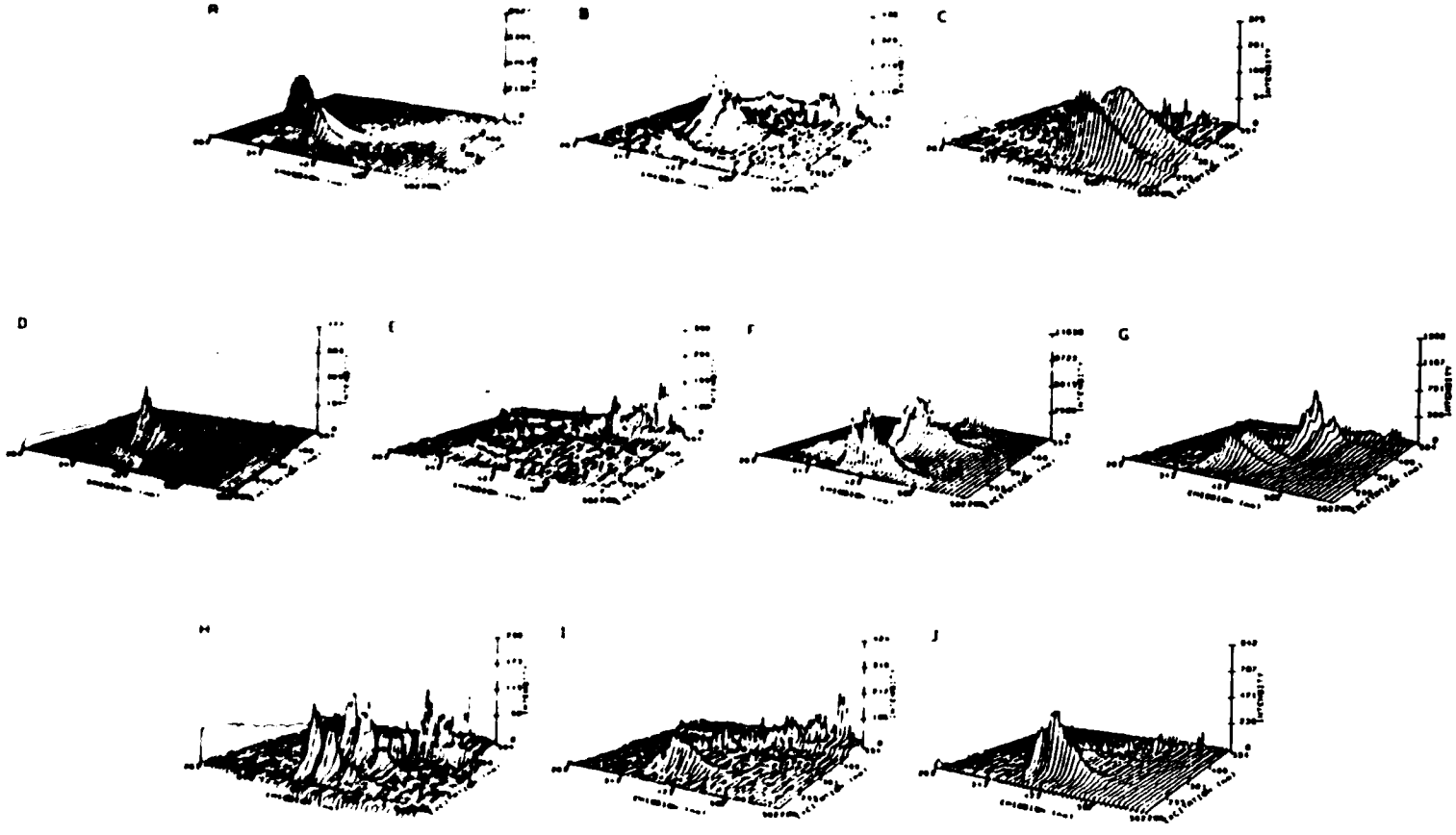
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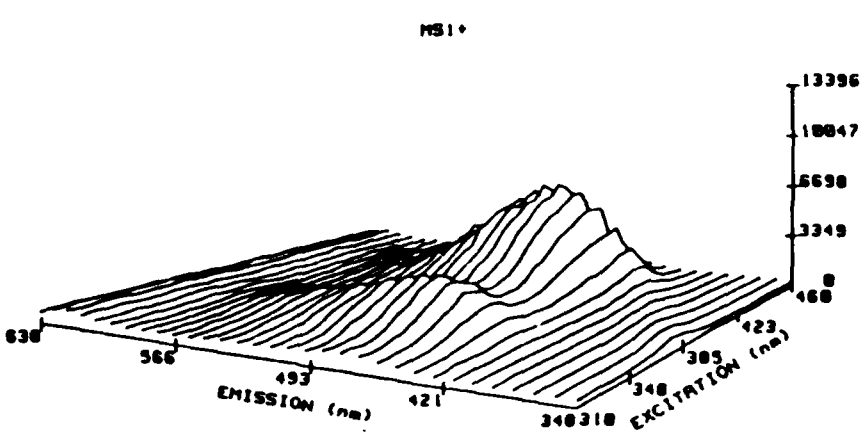
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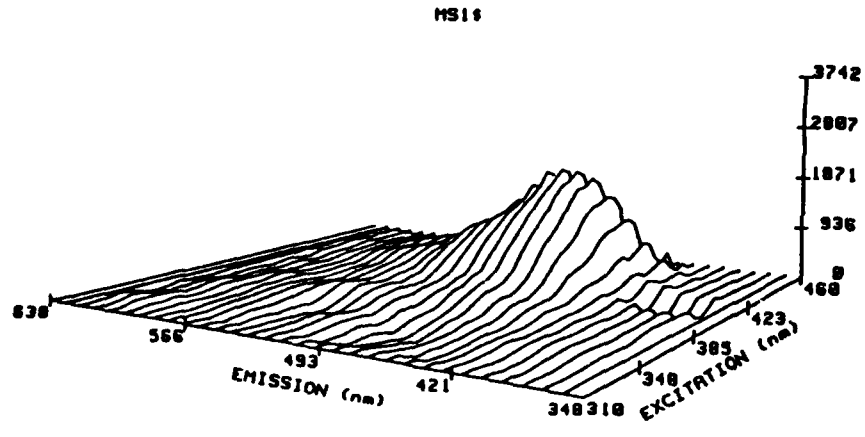
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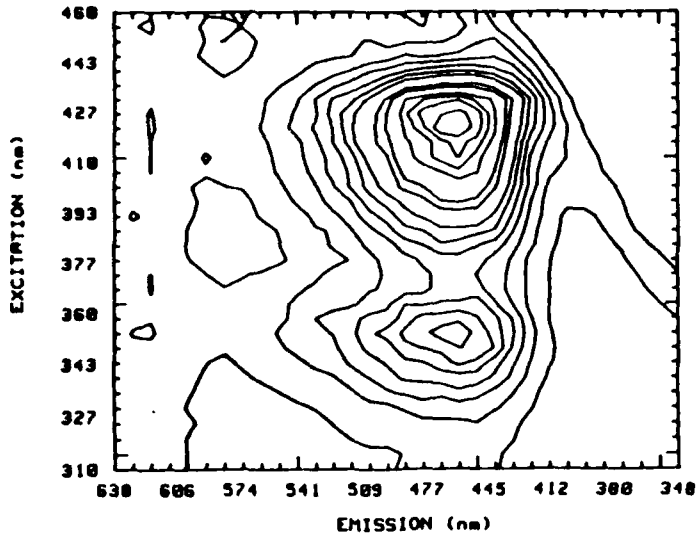
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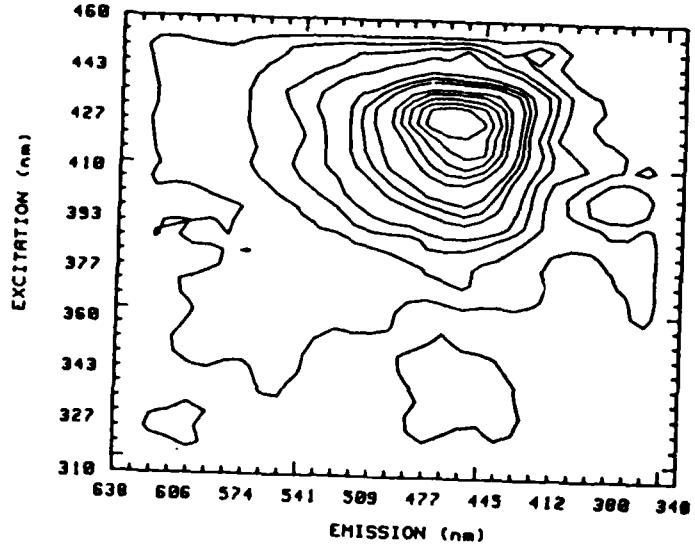
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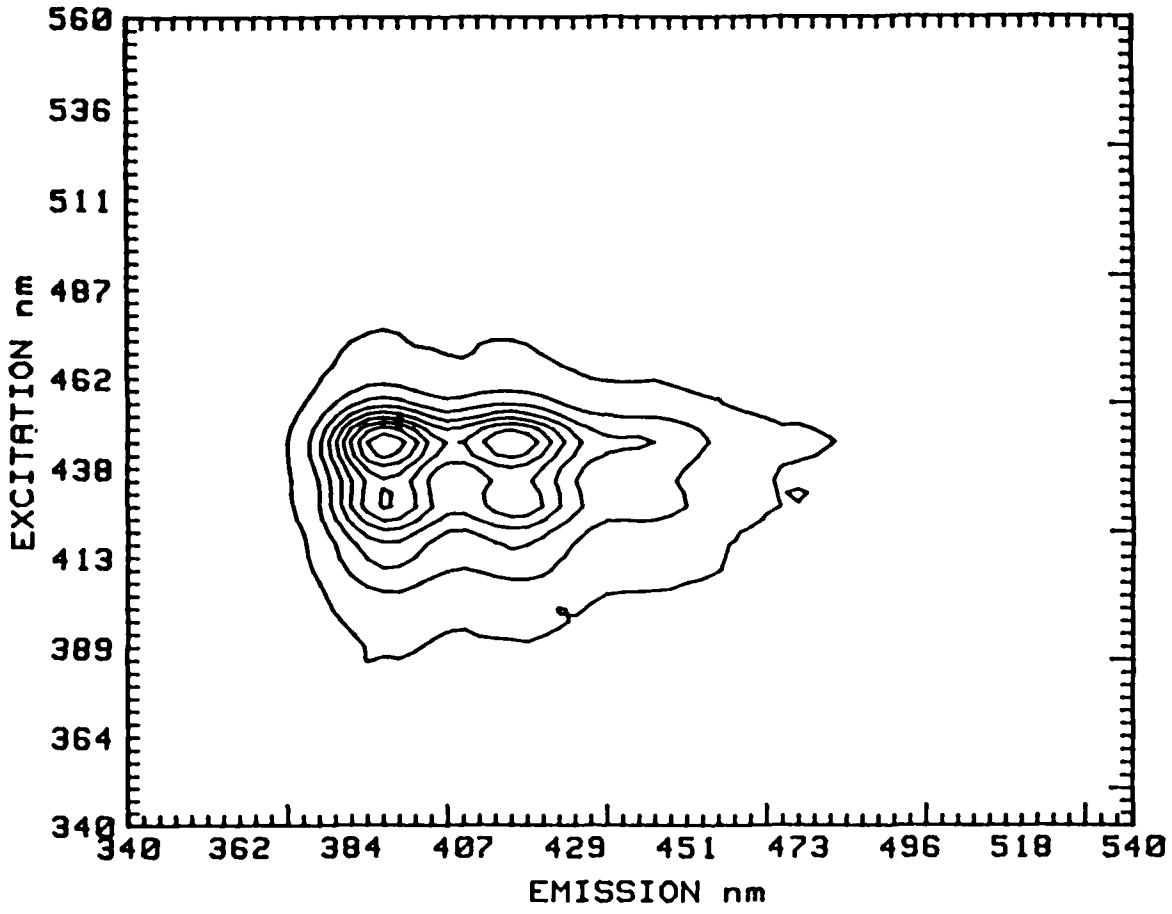
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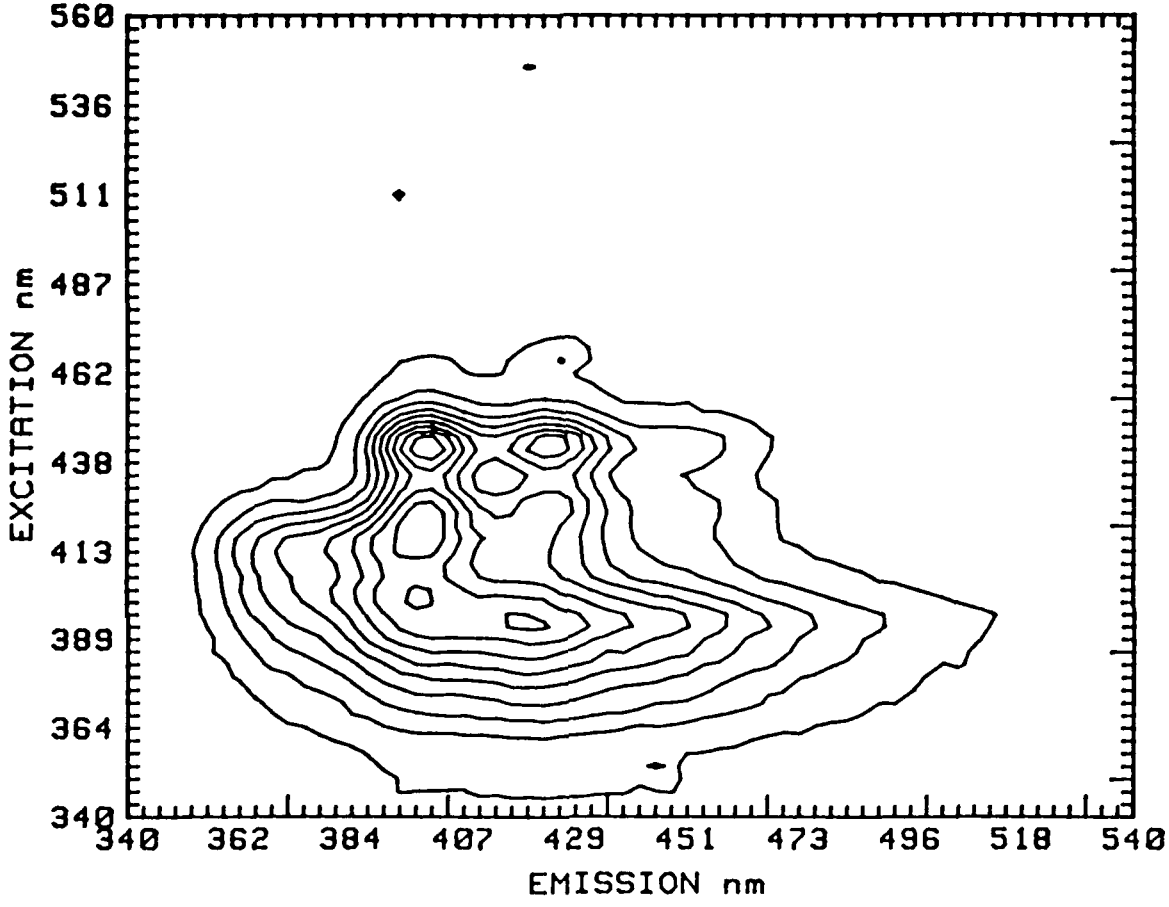
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